REMARKS

The specification has been amended to add reference to applications to which the instant application claims the benefit of priority.

Claims 1-17 have been cancelled and new claim 18 has been added. Support for new claim 18 comes from the application as filed at least at page 11, line 30 - page 12, line 4.

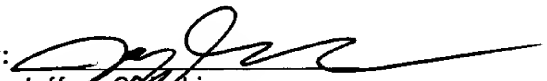
Applicants respectfully request entry of the amendments and examination of the present application with the added claim. If the Examiner has any question regarding the application, he or she is invited to contact the undersigned attorney at the number indicated.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with markings to show changes made."**

Respectfully submitted,

GENENTECH, INC.

Date: August 8, 2001

By: 

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

The paragraph beginning at line 16 page 1 has been amended as follows:

[This is a continuing application under 35 U.S.C. §120/121 of the U.S.S.N. 07/209,665, which is a Continuation-in-Part of U.S.S.N. 07/110,255, filed October 20, 1987 which in turn is a Continuation-in-Part of U.S.S.N. 06/926,977, filed November 4, 1986]. This application is a continuation application under 37 C.F.R. §1.53 (b) (1) claiming priority to co-pending application Serial No. 08/476,837 filed on 7 June 1995, which is a continuation of Serial No. 08/260,662 filed on 16 June 1994, which is a continuation of Serial No. 08/076,280 filed on 11 June 1993, which is a continuation of Serial No. 07/887,575 filed on 18 May 1992, which is a continuation in part of Serial No. 07/237,595 filed on 25 August 1988 which is a continuation in part of Serial No. 07/209,665 filed on 21 June 1988 which is a continuation in part of Serial No. 06/926,977 filed 4 November 1986 which applications are incorporated herein by reference.

In the claims:

Claim 1 has been canceled.

New claim 18 has been added.

[redacted]

SERIAL NUMBER (Series of 1987)	PATENT DATE	PATENT NUMBER
209665		

AL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
17/209,665	06/21/88	514	004	125/186	

DONOGH P. O'BRIEN, HARROW, ENGLAND; GORDON A. VEHAR, SAN CARLOS, CA; JOSIAH N. WILCOX, BELMONT, CA.

CONTINUING DATA********

VERIFIED

THIS APPLN IS A CIP OF 07/110,283 10/20/87 ABN

WHICH IS A CIP OF 06/926,977 11/04/86

FOREIGN/PCT APPLICATIONS********

VERIFIED

ABANDONED

FOREIGN FILING LICENSE GRANTED 10/14/88

Foreign priority claimed / 35 USC 119 conditions met / Verified and Acknowledged (Examiner's Initials)	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
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TITLE

METHOD AND THERAPEUTIC COMPOSITIONS FOR THE TREATMENT OF [MYOCARDIAL INFARCTION] Patients having Angioplasty

PARTS OF APPLICATION FILED SEPARATELY

NOTICE OF ALLOWANCE MAILED	PREPARED FOR ISSUE	CLAIMS ALLOWED

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MAILED JUNE 20, 1988

METHOD AND THERAPEUTIC COMPOSITIONS FOR THE TREATMENT OF MYOCARDIAL INFARCTION

This is a Continuation-in-Part of U.S.S.N. 07/110,283, filed October 20, 1987 which in turn is a Continuation-in-Part of U.S.S.N. 06/926,977, filed November 4, 1986.

Background of the Invention

This invention relates to the treatment of myocardial infarction and more particularly to a therapy capable of preventing the reocclusion of a coronary artery which often accompanies the use of thrombolytic agents in the treatment of myocardial infarction. This invention also relates to the use of tissue factor protein inhibitors to prevent reocclusion of a coronary artery.

The initiating event of many myocardial infarctions (heart attacks) is the hemorrhage into an atherosclerotic plaque. Such hemorrhage often results in the formation of a thrombus (or blood clot) in the coronary artery which supplies the infarct zone (i.e., an area of necrosis which results from an obstruction of blood circulation). This thrombus is composed of a combination of fibrin and blood platelets. The formation of a fibrin-platelet clot has serious clinical ramifications. The degree and duration of the

occlusion caused by the fibrin-platelet clot determines the mass of the infarct zone and the extent of damage.

The primary goal of current treatment for myocardial infarction involves the rapid dissolution of the occluding thrombus and the restoration of blood flow ("reperfusion"). A successful therapy must be capable of eliminating the fibrin-platelet clot in a manner which prevents its reformation after the cessation of therapy. If the fibrin-platelet clot is able to reform, then the affected artery may become reoccluded.

The formation of fibrin-platelet clots in other parts of the circulatory system may be partially prevented through the use of anti-coagulants (such as heparin). Unfortunately, heparin has not been found to be universally effective in preventing reocclusion in myocardial infarction victims in which the degree of blood vessel occlusion (the degree of "stenosis") is greater than or equal to 70%, particularly in those patients with severe residual coronary stenosis.

If an individual has formed a fibrin-platelet clot, the clot may be dissolved through the use of thrombolytic agents. A thrombolytic agent is a medicament capable of lysing the fibrin-platelet thrombus, and thereby permitting blood to again flow through the affected blood vessel. Such agents include streptokinase, prourokinase, urokinase, and tissue-type plasminogen activator (Ganz, W. et al., J. Amer. Coll. Cardiol. 1:1247-1253 [1983]; Rentrop, K.P. et al., Amer. J. Cardiol. 54:29E-31E [1984]; Gold, H.K. et al., Amer. J. Cardiol. 53:122C-125C [1984]).

Treatment with thrombolytic agents can often successfully restore coronary blood flow rapidly enough to interrupt myocardial

infarction. Unfortunately, the dissolved fibrin-platelet clot has been found in a number of patients to reform after cessation of such thrombolytic therapy. This reformation may result in the reocclusion of the affected blood vessels, and is, therefore, of substantial concern (Gold, H.K. et al., supra; Gold H.K. et al., Circulation, 68:150-154 [1983]). Thus, although streptokinase treatment has been found to be successful in dissolving fibrin clots, reocclusion of the affected vessels has been found to occur in approximately 25% of the patients examined (Gold, H.K., et al., Circulation 68:150-154 [1983]).

Tissue-type plasminogen activator (t-PA) is a more desirable thrombolytic agent than either streptokinase or urokinase because it displays greater (though not absolute) specificity for fibrin than does either of these agents (Verstrate, M., et al., Lancet 1:142 [1985]). Tissue-type plasminogen activator (t-PA) is a clot-specific thrombolytic agent with a rapid disposition rate from plasma. Tissue-type plasminogen activator (t-PA has been found to be an effective thrombolytic agent in patients with acute myocardial infarction, producing coronary reflow (i.e., decreasing stenosis) in 45-75 minutes in approximately 70% of patients studied (Gold, H.K. et al., Circulation 73:347 352 [1986]).

Tissue-type plasminogen activator is administered as an infusion at a rate of approximately 1-2 mg/kg patient weight. The benefit of employing t-PA is significantly offset by the spontaneous rate of acute reocclusion which follows the cessation of t-PA therapy. It has been observed that cessation of t-PA therapy resulted in reocclusion of affected blood vessels in approximately 45% of patients studied (Circulation 73:347-352 [1986]). Increased t-PA dosages have not been found to decrease the tendency for coronary artery reocclusion. Significantly, the

possibility of thrombin clot reformation is closely related to the degree of residual coronary stenosis (i.e., the extent of blood vessel blockage). Thus, reocclusion is more probable in individuals in which high grade stenosis (i.e., greater than 70% quantitative stenosis or greater than 80% non-quantitative stenosis) has occurred. The reocclusion of blood vessels has been found to be inhibited by continued infusion of t-PA (Gold, H.K. et al., Circulation 73:347-352 [1986]).

The general mechanism of blood clot formation is reviewed by Ganong, W.F. (In: Review of Medical Physiology, 9th ed., Lange, Los Altos, CA, pp 411-414 [1979]). Blood coagulation performs two functions; the production of thrombin which induces platelet aggregation and the formation of fibrin which renders the platelet plug stable. A number of discrete proenzymes and procofactors, referred to as "coagulation factors", participate in the coagulation process. The process consists of several stages and ends with fibrin formation. Fibrinogen is converted to fibrin by the action of thrombin. Thrombin is formed by the proteolytic cleavage of a proenzyme, prothrombin. This proteolysis is effected by activated factor X (referred to as factor X_a) which binds to the surface of activated platelets and in the presence of Va and ionic calcium cleaves prothrombin.

Activation of factor X may occur by either of two separate pathways, the extrinsic or the intrinsic. The intrinsic cascade consists of a series of reactions wherein a protein precursor is cleaved to form an active protease. At each step, the newly formed protease will catalyze the activation of another protease at the subsequent step of the cascade. A deficiency of any of the proteins in the pathway blocks the activation process at that step, thereby preventing clot formation and typically gives rise to a

tendency to hemorrhage. Deficiencies of factor VIII or factor IX, for example, cause the severe bleeding syndromes haemophilia A and B, respectively. In the extrinsic pathway of blood coagulation, tissue factor, also referred to as tissue thromboplastin, is released from damaged cells and facilitates factor X in the presence of factor VII and calcium. Although activation of factor X was originally believed to be the only reaction catalyzed by tissue factor and factor VII, it is now known that an amplification loop exists between factor X, factor VII, and factor IX (Osterud, B., and S.I. Rapaport, Proc. Natl. Acad. Sci. USA 74:5260-5264, 1977; Zur, M. et al., Blood 52: 198, 1978). Each of the serine proteases in this scheme is capable of converting by proteolysis the other two into the activated form, thereby amplifying the signal at this stage in the coagulation process (Figure 2). It is now believed that the extrinsic pathway may in fact be the major physiological pathway of normal blood coagulation (Haemostasis 13:150-155 1983). Since tissue factor is not normally found in the blood, the system does not continuously clot; the trigger for coagulation would therefore be the release or exposure of tissue factor from damaged tissue, e.g. atherosclerotic plaque.

Tissue factor is an integral membrane glycoprotein which, as discussed above, can trigger blood coagulation via the extrinsic pathway. Bach, R. et al., J. Biol Chem. 256(16), 8324-8331 (1981). Tissue factor consists of a protein component (previously referred to as tissue factor apoprotein-III) and a phospholipid. Osterud, B. and Rapaport, S.I., PNAS 74, 5260-5264 (1977). The complex has been found on the membranes of monocytes and different cells of the blood vessel wall. Osterud, B., Scand. J. Haematol. 32, 337-345 (1984). Tissue factor from various organs and species has been reported to have a relative molecular mass of 42,000 to 53,000. Human tissue thromboplastin has been described as consisting of a

tissue factor protein inserted into phospholipid bilayer in an optimal ratio of tissue factor protein:phospholipid of approximately 1:80. Lyberg, T. and Prydz, H., Nouv. Rev. Fr. Hematol. 25(5), 291-293 (1983). Purification of tissue factor has been reported from various tissues such as,: human brain (Guha, A. et al. PNAS 83, 299-302 [1986] and Broze,G.H. et al., J.Biol.Chem. 260[20], 10917-10920 [1985]); bovine brain (Bach, R. et al., J. Biol. Chem. 256, 8324-8331 [1981]); human placenta (Bom, V.J.J. et al., Thrombosis Res. 42:635-643 [1986]; and, Andoh, K. et al., Thrombosis Res. 43:275-286 [1986]); ovine brain (Carlsen, E. et al., Thromb. Haemostas. 48[3], 315-319 [1982]); and, lung (Glas, P. and Astrup, T., Am. J. Physiol. 219, 1140-1146 [1970]. It has been shown that bovine and human tissue thromboplastin are identical in size and function. See for example Broze, G.H. et al., J. Biol. Chem. 260(20), 10917-10920 (1985). It is widely accepted that while there are differences in structure of tissue factor protein between species there are no functional differences as measured by in vitro coagulation assays. Guha et al. supra. Furthermore, tissue factor isolated from various tissues of an animal, e.g. dog brain, lung, arteries and vein was similar in certain respects such as, extinction coefficient, content of nitrogen and phosphorous and optimum phospholipid to lipid ratio but differed slightly in molecular size, amino acid content, reactivity with antibody and plasma half life. Gonmori, H. and Takeda, Y., J. Physiol. 229(3), 618-626 (1975). All of the tissue factors from the various dog organs showed clotting activity in the presence of lipid. Id. It is widely accepted that in order to demonstrate biological activity, tissue factor must be associated with phospholipids. Pitlick, F.A., and Nemerson, Y., Biochemistry 9, 5105-5111 (1970) and Bach,R. et al. supra. at 8324. It has been shown that the removal of the phospholipid component of tissue factor, for example by use of a phospholipase, results in a loss of its biological

activity. Nemerson, Y., J.C.I. 47, 72-80 (1968). Relipidation can restore in vitro tissue factor activity. Pitlick, F.A. and Nemerson, Y. Biochemistry 9, 5105-5113 (1970) and Freyssinet, J.M. et al., Thrombosis and Haemostasis 55, 112-118 [1986].

Infusion of tissue factor has long been believed to compromise normal haemostasis. In 1834 the French physiologist de Blainville first established that tissue factor contributed directly to blood coagulation. de Blainville, H. Gazette Medicale Paris, Series 2, 524 (1834). de Blainville also observed that intravenous infusion of a brain tissue suspension caused immediate death which he observed was correlated with a hypercoagulative state giving rise to extensively disseminated blood clots found on autopsy. It is now well accepted that intravenous infusion of tissue thromboplastin induces intravascular coagulation and may cause death in various animals. (Dogs: Lewis, J. and Szeto I.F., J. Lab. Clin. Med. 60, 261-273 (1962); rabbits: Fedder, G. et al., Thromb. Diath. Haemorrh. 27, 365-376 (1972); rats: Giercksky, K.E. et al., Scand. J. Haematol. 17, 305-311 (1976); and, sheep: Carlsen,E. et al., Thromb. Haemostas. 48, 315-319 [1982]).

In addition to intravascular coagulation or a hypercoagulative state resulting from the exogenous administration of tissue factor, it has been suggested that the intravascular release of tissue thromboplastin may initiate disseminated intravascular coagulation (DIC). Prentice, C.R., Clin. Haematol. 14(2), 413-442 (1985). DIC may arise in various conditions such as shock, septicaemia, cardiac arrest, extensive trauma, bites of poisonous snakes, acute liver disease, major surgery, burns, septic abortion, heat stroke, disseminated malignancy, pancreatic and ovarian carcinoma, promyelocytic leukemia, myocardial infarction, neoplasms, systemic lupus erythematosus, renal disease and

eclampsia. Present treatment of DIC includes transfusion of blood and fresh frozen plasma; infusion of heparin; and removal of formed thrombi. The foregoing clinical syndromes suggest that endogenous release of tissue factor can result in severe clinical complications. Andoh, K. et al., Thromb. Res. 43, 275-286 (1986). Efforts were made to overcome the thrombotic effect of tissue thromboplastin using the enzyme thromboplastinase. Gollub, S. et al., Thromb. Diath. Haemorh. 7, 470-479 (1962). Thromboplastinase is a phospholipase and would presumably cleave the phospholipid portion of tissue factor. Id.

An object of the present invention is to provide an effective therapy for myocardial infarction which limits necrosis by permitting early reperfusion and by preventing reocclusion.

A further object of this invention is to provide a therapeutic composition for treatment of myocardial infarction and prevention of reformation of fibrin-platelet clots, i.e. reocclusion.

Yet another object of this invention is to provide an anticoagulant therapeutic, that is an antagonist to tissue factor protein, to neutralize the thrombotic effects of endogenous release of tissue thromboplastin which may result in a hypercoagulative state. Particularly, such an anticoagulant, that is an antagonist to tissue factor protein, would neutralize the hypercoagulant effects of endogenously released tissue thromboplastin by inactivating tissue factor protein. Such a tissue factor protein antagonist can be an antibody or other protein that specifically inactivates the protein component.

Summary of the Invention

This invention is based in part on the novel and unexpected observation that tissue factor was found to be present in atherosclerotic plaques. It was observed that tissue factor was present in the plaque in greater amounts than in normal vessels. It was also observed that tissue factor mRNA was present in both mesenchymal like intimal cells as well as in macrophages and cells adjacent to the cholesterol clefts within the atherosclerotic plaque.

Accordingly, in one aspect the invention is directed to administration of a pharmaceutical composition comprising a tissue factor protein inhibitor and a thrombolytic agent. In another aspect the invention provides a method of treatment following angioplasty or for myocardial infarction which comprises administering to a patient in need of such treatment:

a) a tissue factor protein inhibitor capable of preventing potential clot reformation, in a therapeutically effective amount to prevent such reformation; in combination with

b) a thrombolytic agent, in a therapeutically effective amount to dissolve a fibrin-platelet clot.

A further aspect of this invention is directed to an anticoagulant to neutralize the coagulant effects of endogenously released tissue thromboplastin by inactivating tissue factor protein. Yet another aspect of this invention is directed to an anti-atherosclerotic agent to neutralize the effects of endogenously released tissue thromboplastin in the formation of atherosclerotic plaques by inactivating tissue factor protein.

Brief Description of the Drawings

Figure 1a-c (herein referred to as Figure 1). Localization of tissue factor in the normal human saphenous vein. Cells containing tissue factor protein were detected by immunocytochemistry using the Vectastain alkaline phosphatase method (positive cells stain red). Scattered cells in the tunica media were lightly stained by the RD010 antibody whereas endothelial cells lining the normal vessel were always negative (panel A magnification 500X). Strong immunohistochemical staining was always seen in the adherent adventitial fibroblasts (panel B, magnification 500X). In situ hybridization using a specific ^{35}S-labelled tissue factor mRNA probe confirmed that there were scattered tissue factor producing cells in the media (panel C, magnification 310X) and adventitia (not shown).

Figure 2a-c (herein referred to as Figure 2). Localization of tissue factor in the human atherosclerotic plaque by in situ hybridization and immunohistochemistry. Carotid endarterectomy specimens were hybridized to an ^{35}S-labelled tissue factor mRNA probe (panel A, magnification 125X) and revealed many cells producing tissue factor in the atherosclerotic plaque. Immunohistochemistry with tissue factor antibody RD010 indicated strong staining of the necrotic core region of the plaque particularly in areas adjacent to the cholesterol clefts that was not entirely cell associated (panel B, magnification 125X). In situ hybridization of serial sections indicated that there were cells containing tissue factor mRNA adjacent to the cholesterol clefts (panel C, magnification 310X) suggesting local synthesis of the tissue factor protein detected in this region.

Figure 3a-b (herein referred to as Figure 3). Localization of tissue factor protein in macrophage foam cell regions of the

atherosclerotic plaque by immunohistochemistry (panel A, magnification 125X; panel B, magnification 500X).

Figure 4. Procoagulant activity of a carotid endarterectomy specimen measured using a modified one-stage prothrombin time assay in Factor XII deficient plasma. The procoagulant activity of the tissue was significantly reduced by preincubation with a neutralizing polyclonal antibody RD010.

Detailed Description

As used herein, the term "tissue factor protein antagonist" refers to a substance which inhibits or neutralizes the procoagulant activity of tissue factor. Such antagonists accomplish this effect in various ways. First, one class of tissue factor protein antagonists will bind to tissue factor protein with sufficient affinity and specificity to neutralize tissue factor protein such that it cannot bind to factor VII or VII_a nor effect the proteolysis of factors IX or X when in complex with factor VII or VII_a. Included within this group of molecules are antibodies and antibody fragments (such as, for example, F(ab) or $F(ab')_2$ molecules). Another class of tissue factor antagonists will neutralize tissue factor protein by creating a complex of molecules, e.g., the naturally occurring tissue factor inhibitor "lac" which comprises lipoprotein associated coagulation inhibitor which forms an inactive complex of tissue factor, factor VII, factor X and phospholipid (Broze, G.J. et al., PNAS 84:1886-1890 [1987]). Another class of tissue factor protein antagonists are fragments of tissue factor protein or small molecules, i.e. peptidomimetics, which possess the structural conformation of that part of tissue factor protein which binds to factor VII to activate factor IX. Yet another class of tissue factor protein antagonists will inactivate tissue factor protein or the tissue factor/factor

VII_a complex by cleavage, e.g. a specific protease. A fifth class of tissue factor protein antagonists block the binding of tissue factor protein to factor VII, e.g., a factor VII antibody directed against a domain of factor VIII which is involved in the activation of factor VII by tissue factor.

Tissue factor protein antagonists are useful in the treatment of myocardial infarction to prevent reocclusion or in the therapy of various coagulation disorders as evidenced by altered plasma fibrinogen levels as described herein e.g. DIC occurring during severe infections and septicemias, after surgery or trauma, instead of or in combination with other anticoagulants such as heparin.

An example of an antagonist which will neutralize tissue factor protein is an antibody to tissue factor protein. Tissue factor protein neutralizing antibodies are readily raised in animals such as rabbits or mice by immunization with tissue factor protein in Freund's adjuvant followed by boosters as required. Immunized mice are particularly useful for providing sources of B cells for the manufacture of hybridomas, which in turn are cultured to produce large quantities of inexpensive anti-tissue factor protein monoclonal antibodies. Such tissue factor protein monoclonal antibodies have been prepared by Carson, S.D. et al., Blood 66(1), 152-156 (1985).

As used herein, "tissue factor protein" refers to a protein capable of correcting various bleeding disorders particularly those associated with deficiencies in coagulation factors. Tissue factor protein is distinct from tissue factor or tissue thromboplastin in that it lacks the naturally occurring lipid portion of the molecule. Tissue factor protein also includes tissue factor

protein associated with phospholipid which lipid is distinct from the naturally occurring lipid associated with tissue thromboplastin and which displays coagulation-inducing capability without the concomitant toxicity observed with the lipidated protein.

Tissue factor is released from damaged cells and activates factors IX and X in the presence of factor VII or VII_a and calcium. The activation of factor X by the extrinsic pathway of coagulation has an absolute requirement for tissue factor. Silverberg, S.A., et al., J. Biol. Chem. 252, 8481-8488 (1977). Until the discovery of this invention, the cellular distribution of tissue factor protein producing cells within tissues from which tissue factor had been isolated was unknown. Nor was it known that tissue factor protein is present and exposed in atherosclerotic plaques. Atherosclerotic plaques obtained from carotid endarterectomy surgery were examined for tissue factor mRNA and protein. Extensive mRNA hybridizations were seen in several regions of atherosclerotic plaque (figure 2a). Multiple plaques were screened and cells showing positive hybridization were seen in all of them. It was possible to confirm that immunohistochemistry and in situ hybridization labelled the same cells on serial sections.

The necrotic cores of the plaques were characterized by extensive tissue factor protein localization in the extracellular matrix particularly surrounding cholesterol clefts (figure 2b). Additional protein staining was also seen in the macrophage-rich foam cell regions of at least one atherosclerotic plaque (figure 3). Such foam cell-rich regions often lay underneath the fibrous cap adjacent to the necrotic cores. Finally, as with the normal vessels, no tissue factor mRNA or protein was detected in surface or capillary endothelium.

The tissue factor protein detected by immunocytochemistry was actively thrombogenic as determined in coagulation assays. Preincubation of the tissue with factor XII deficient plasma accelerated the coagulation time over that with serum alone.

This invention encompasses the treatment of myocardial infarction through the administration of a tissue factor protein antagonist and a thrombolytic agent.

The term "thrombolytic agent" is meant to refer to any agent capable of either dissolving a fibrin-platelet clot, or inhibiting the formation of such a clot. Examples of thrombolytic agents include streptokinase, prourokinase, urokinase, and tissue-type plasminogen activator ("t-PA"). Although natural t-PA (Collen, et al., EP application publication no. 041,766, filed 6/10/81) may be employed, it is preferable to employ recombinant t-PA (Goeddel et al. EP application publication no. 093,619, filed 5/4/83). The invention may additionally employ hybrids, physiologically active fragments or mutant forms of the above thrombolytic agents. The term "tissue-type plasminogen activator" as used herein is intended to include such hybrids, fragments and mutants, as well as both naturally derived and recombinantly derived tissue-type plasminogen activator.

The tissue factor protein antagonist and the thrombolytic agent of the present invention are intended to be provided to the recipient in combination. Medicaments are considered to be provided "in combination" with one another if they are provided to the patient concurrently, or if the time between the administration of each medicament is such as to permit an overlap of biologic activity. It is preferable to provide the tissue factor protein

antagonist to the patient prior to the administration of the thrombolytic agent.

The tissue factor protein antagonist of the present invention is provided with a goal of preventing potential reformation of fibrin-platelet clots. Such fibrin-platelet clots may form as a consequence of the cessation of treatment with a thrombolytic agent. Advanced human atherosclerosis is characterized by intimal smooth muscle cell proliferation accompanied by accumulation of fats and inflammatory cells including macrophages and T cells within the atherosclerotic plaque (Ross,R., N.Engl. J. Med. 314:488-500 [1986]; Gown et al., Am. J. Pathol. 125:191-207 [1986]; Jonasson et al., Arteriosclerosis 6:131-138 [1986]). Thrombosis is commonly the critical event which converts an asymptomatic atherosclerotic plaque into a symptomatic one (Falk,E. Br. Heart J. 50:127-134 [1983]; Sherman et al., N. Engl. J. Med. 315:913-919 [1986]; Impesato, A.M. et al., Ann. Surg. 197:195-203 [1983]) whereas non-diseased arteries hardly ever become thrombosed. It has been suggested that plaque rupture is the integral event that precipitates clot formation (Forrester et al., Circulation 75:505-513 [1987]). An occlusive mural thrombus accompanies most cases of acute myocardial infarctions (Buja, L.M. et al., Am. J. Cardiol. 47:343-356 [1981]; Horie, T. et al., Brit. Heart. J. 40:153-161 [1978]). Plaque rupture or cracking is normally found to underlie such thrombi, and in many cases the thrombus is seen to extend into the region of the necrotic core of the plaque extruding through such cracks. This is true of both the coronary (Falk, Br. Heart J. 50:127-134 [1983]; Chapman,I, Arch. Path. 30:256-261 [1965]; Drury, J. Path. Bact. 67:207-215 [1954]) and cerebral arteries (Constantinides, J. Arch. Pathol. 83:422-428 [1967]). The source of the thrombogenicity of the plaque has not previously been determined but was previously assumed to occur when

blood components come into contact with fats or the collagen matrix within the plaque. The current studies establish that there is a) significant synthesis of tissue factor protein in atherosclerotic plaques; b) that tissue factor protein accumulates in the necrotic core and is found in foam cell rich regions of the plaque; and c) that there is in the plaque procoagulant activity due to tissue factor by in vitro coagulation assays that is significantly reduced by tissue factor protein antibodies. These results indicate that overproduction and/or trapping of tissue factor protein in the atherosclerotic plaque may play a significant role in thrombosis associated with human atherosclerotic vessels and clot reformation following thrombolytic therapy in the treatment of myocardial infarction.

An amount of tissue factor protein antagonist capable of preventing partial reformation of a clot when provided to a patient is a "therapeutically effective" amount. In order to prevent potential clot reformation tissue factor protein antagonist will be provided using an amount per kilogram of patient weight determined by the ordinarily skilled physician. This dosage may be administered, in one embodiment, over a period of between 75-180 minutes, by continual intravenous infusion. The tissue factor protein antagonist may be given by cardiac catheterization or by an intravenously injectable bolus at a dose of about in the range of 0.01-4.0 milligrams per kilogram of patient weight. If the tissue factor protein antagonist is provided by an intravenously injected bolus, a single bolus may be sufficient to prevent potential clot reformation. Although the tissue factor protein antagonist of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus, it is preferable to prepare such a bolus by dissolving the tissue factor protein antagonist in saline.

The thrombolytic agent is provided in order to cause the lysis of an occluding thrombus. An amount of thrombolytic agent capable of causing such lysis is a "therapeutically effective" amount. The thrombolytic agent of the present invention is preferably provided at a dose of between 0.01-2.5 mg per kg of patient weight. In one embodiment, the thrombolytic agent is provided over a prolonged period (i.e., from about 60 to about 120 minutes). In a preferred embodiment, the thrombolytic agent of the present invention is provided as an intravenously injected bolus containing between 0.01-1.0 mg/kg, and most preferably between 0.1-1.0 mg/kg. The thrombolytic agent of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus. It is, however, preferable to prepare such a bolus by dissolving the thrombolytic agent in an appropriate buffer.

A patient treated according to the preferred embodiment will, therefore, receive an intravenously injected bolus of the tissue factor protein antagonist in combination with an intravenously injected bolus of the thrombolytic agent. Importantly, the use of the preferred treatment results in the dissolution of the occluding thrombus at a rate which greatly exceeds the rate of thrombus dissolution when either the tissue factor protein antagonist or the thrombolytic agent is provided by infusion. Additionally, the risk of reocclusion is substantially reduced. A patient treated according to the preferred embodiment may not require heparin which is generally required with a maintenance infusion t-PA treatment.

These unexpected findings provide a method of treatment in which the administration of a bolus of a tissue factor protein

antagonist in combination with the administration of a bolus of a thrombolytic agent are capable of dissolving an occluding thrombus and minimizing the risk of reocclusion

As would be apparent to one of ordinary skill in the art, the required dosage of the tissue factor protein antagonist or thrombolytic agent will depend upon the severity of the condition of the patient, and upon such criteria as the patient's height, weight, sex, age, and medical history.

The tissue factor protein antagonist or thrombolytic agent of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in Remington's Pharmaceutical Sciences (16th ed., Osol, A. (ed.), Mack, Easton, PA [1980]). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the tissue factor protein antagonist or thrombolytic agent, either alone, or with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved by the use of polymers to complex or adsorb the tissue factor protein antagonist or thrombolytic agents of the present invention. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the concentration of such macromolecules. Another possible method for

controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agent in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in Remington's Pharmaceutical Sciences (1980).

The thrombolytic agent or tissue factor protein antagonist may be provided to a patient by means well known in the art. Such means of introduction include oral means, intranasal means, subcutaneous means, intramuscular means, intravenous means, intra-arterial means, or parenteral means. In the most preferred method of treatment for myocardial infarction, a patient is provided with a bolus (intravenously injected) at a dosage determined by the ordinarily skilled physician taking into account various criteria which establish that particular patient's clinical condition.

Having now generally described this invention, it will be better understood by reference to certain specific examples which are included herein for purposes of illustration only, and are not intended to limit the invention, unless specified.

EXAMPLE 1

General Materials and Methods

Triton X-100 was from Calbiochem, San Diego, CA. All chemicals and reagents for preparative and analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories. Richmond, CA. Factor IX_a/Factor X reagent and S2222/I2581 were obtained from Helena Laboratories (Kabi Coatest kit, Helena Laboratories, Beaumont, CA., Catalogue No. 5293). YM 10 ultrafiltration membranes were from Amicon. Factor VII was purchased from Sigma Chemical. Crude phosphotidylcholine (lecithin granules from soya bean) were obtained from Sigma, St. Louis, MO. All other chemicals were of reagent grade or better.

Purification of Tissue Factor Protein

Tissue factor protein was purified using immunoaffinity purification using an IgG monoclonal antibody that binds human tissue factor protein.

Human tissue factor protein was synthesized in recombinant culture as described in European Patent Application No. 88301190.0, filed 2/12/88. The following immunogens were injected into a BALB/c mouse (29.1.B) according to the schedule described below: recombinant human tissue factor protein (rTF) (0.72 mg/ml having a specific activity 4687 U/mg) OR (.07 mg/ml having a specific activity 17040 U/mg); recombinant tissue factor protein obtained from a tissue factor-gD fusion cleaved by thrombin to remove the herpes-gD sequences from the amino terminal end (rTF:gDThr) (4300 U/ml) and recombinant tissue factor-herpes-gD fusion (rTF-gD) (approximately 740 U/ml) on the following immunization schedule:

Day	Administration Route	Immunogen
1.	subcutaneous (sc)	0.25 ml of r-TF in Freund's complete adjuvant
14.	half sc and half intraperitoneal (ip)	0.25 ml r-TF in incomplete Freund's adjuvant
28.	I.P.	0.25 ml of r-TF in PBS
42.	I.P.	0.25 ml of r-TF in PBS
62.	I.P.	0.25 ml of r-TF in PBS
75.	I.P.	0.52 ml of r-TF-gD in PBS
85.	I.p.	0.5 ml of r-TF-gDThr in PBS

The anti-TF titer assayed by radio-immunoprecipitation (RIP) and ELISA increased gradually throughout the immunizations to day 85.

The RIP assay used 0.005 ml of sera from immunized and non-immunized mice diluted with 0.495 ml of PBSAT (PBS containing 0.5% bovine serum albumin [BSA] and 0.1% Triton X-100). 50,000 cpm of ^{125}I-rTF was added and the mixture was incubated for 2 hr at room temperature. ^{125}I-rTF complexed with antibody was precipitated by incubating for 1 hr at room temperature with 0.05 ml of SPA beads. The SPA beads consisted of staphylococcal protein A bound to sepharose CL-4B beads that had been pre-incubated with rabbit anti-mouse IgG and stored in PBS, 0.1% BSA and 0.02% NaN_3. The beads were pelleted, washed three times with PBSAT and counted in a gamma counter.

The ELISA consisted of 0.1 ml of rTF (0.5 μg/ml) in carbonate buffer pH 9.6 adsorbed to microtiter wells for 2 hr at 37°C. Further non-specific adsorption to the wells was blocked for

1 hr at 37°C with PBSA (PBS containing 5% BSA). The wells were washed 3 times with PBST (PBS containing 0.1% Tween 20) and the serum samples diluted in PBSAT was added and incubated 2 hrs. at 22°C. The wells were washed 3 times with PBSAT. 0.1 ml of goat anti-mouse immunoglobulin conjugated to horseradish peroxidase was added to each well and incubated for 1 hr at room temperature. The wells were washed again and o-phenylenediamine dihydrochloride was added as substrate and incubated for 25 minutes at room temperature. The reaction was stopped with 2.5 M H_2SO_4 and the absorbance of each well was read at 492 nm.

On day 89 the spleen from mouse 29.1.B was harvested, disrupted and the spleen cells fused with X63-Ag8.653 (ATCC CRL 1580) non-secreting mouse myeloma cells using the PEG fusion procedure of S. Fazakas de St. Groth et al., J. Immun. Meth., 35:1-21 (1980). The fused culture was seeded into 4 plates each containing 96 microtiter wells and cultured in HAT (hypoxanthine, aminopterin and thymidine) media by conventional techniques (Mishell and Shiigi, Selected Methods in Cellular Immunology, W.H. Freeman & Co., San Francisco, pp. 357-363 [1980]). The anti-TF activity of culture supernatants was determined by ELISA and RIA. Twelve stable fusions (hybridomas) secreted anti-TF as determined by ELISA or antigen capture RIA described below. The hybridomas were expanded and cloned by limiting dilution using published procedures (Oi, V.J.T. & Herzenberg, L.A., "Immunoglobulin Producing Hybrid Cell Lines" in Selected Methods in Cellular Immunology, p. 351-372, Mishell, B.B. and Shiigi, S.M. [eds.], W.H. Freeman & Co. [1980]). Selection of clones was based on: macroscopic observation of single clones, ELISA and RIA. The antibody was isotyped using a Zymed isotyping kit according to the accompanying protocol (Zymed Corp.) Large quantities of specific monoclonal antibodies were produced by injection of cloned

hybridoma cells in pristane primed mice to produce ascitic tumors. Ascites were then collected and purified over a protein-A Sepharose column.

Antigen capture RIA methodology used ^{125}I labeled tissue factor protein with the lactoperoxidase-enzymobeads (BIORAD, Richmond, CA.) following the vendor's suggested protocol. Polystyrene "strip wells" were coated with 100 µl/well of goat anti-mouse IgG (H & L chain specific, Boehringer Mannheim) at 5 µg/ml in pH 9.6 carbonate buffer for 1 hour at 37°C. The strips were washed with PBSAT and incubated with 50,000 CPM ^{125}I-TF in 100 µl BPBST for 2 hours at 22°C. The strips were washed and individual wells were counted on a 20/20 gamma counter to determine percentage of input counts bound.

The foregoing method for immunization and screening for anti-tissue factor antibody is exemplary. For example, immunization could be carried out using a particular antigen such as r-tissue factor protein, gD-tissue factor fusion or thrombin cleaved gD-tissue factor fusion. The immunization protocol could be modified by altering the route of administration, the method of in vitro immunization, various conjugation or adjuvant techniques or by selecting from various available sources species of B cells. Antibody could be screened for neutralization of tissue factor activity using, for example, the chromogenic assay described below. Screening for neutralizing antibodies could be carried out by testing the harvested supernatant in the chromogenic assay rather than using an ELISA or RIA.

Approximately 5 ml of ascites fluid was centrifuged at 3000 rpm in a Sorvall 6000 at 4°C for 10 min. The clear layer of pristane and the layer of lipid was removed with a pasteur pipet.

The ascites fluid was transferred to a 50 ml centrifuge tube. The ascites fluid was sterile filtered through a 0.45 M filter. 1.11 gram of KCl was added to the ascites to yield a final concentration of 3M KCl.

The ascites was loaded onto a 10 ml column containing SPA Sepharose (Fermentech). The column was washed with 3M KCl. The mouse IgG was eluted with 3 to 4 column volumes of 0.1 M acetic acid in 0.15 M NaCl pH 2.8.

The antibody D3 was coupled to CNBr Sepharose according to the manufacturer's instructions at 3 mg IgG per ml of Sepharose. (See Pharmacia Co. instruction manual). Transfected 293S cells were grown in a 1:1 mixture of Ham's F-12 (w/o glycine, hypoxanthine and thymidine) and DMEM (w/o glycine). Additions to the basal medium include: 10% dialysed or diafiltered fetal calf serum, 50 nM methotrexate, 2.0 mM L-glutamine and 10 mM HEPES buffer.

A frozen vial of 293S (63/2S CISTF) is thawed in a tissue culture flask containing the described medium. When the culture reaches confluency it is trypsinized with trypsin-EDTA mixture and a small portion of the cell population was used to inoculate larger flasks. Cultures were monitored daily by phase microscopy to determine growth (percent confluency), morphology and general health. When rollerbottle cultures were confluent (usually within 5-7 days), the cells were trypsinized and counted. Cells were enumerated and their viabilities determined by the trypan blue exclusion technique. Typical cell numbers from a confluent 850 cm^2 rollerbottle were between 1 to 4 x 10^8 cells. Cells were suspended in 0.01 M sodium phosphate, 0.15 M NaCl. Cells were collected by centrifugation at 5000 rpm. Cells were resuspended in 50 mls TBS

containing 1% Triton X per flask. Cultures were incubated one hour at room temperature and then centrifuged 8000 x g for 20 min. Supernatant was loaded onto the D3-Sepharose column described above. The column was washed and eluted with .1 M acetic acid, 150 mM NaCl and .05% Tween 80.

Assay for Tissue Factor Protein

1. Chromogenic tissue factor assay.

All were relipidated prior to assay. As discussed above tissue factor has an absolute requirement for phospholipid to exhibit activity in in vitro assay systems (Pitlick and Nemerson, Supra). Lecithin granules were homogenized in Tris 0.05 M, 0.1 M NaCl pH7.4 (TBS) containing 0.25% sodium deoxycholate to a concentration of 1 mg/ml. This solution (PC/DOC) was used to relipidate tissue factor as follows. Tissue factor protein was diluted into TBS containing 0.1% bovine serum albumin (TBSA). Fifty microliters were placed in a 12x75mm polystyrene test tube and 50 μl PC/DOC solution was added. Three hundred and fifty (350) microliters TBSA were then added along with 25 μl 100 mM $CdCl_2$. This relipidation mixture was allowed to incubate at 37°C for 30 min.

For the chromogenic assay, relipidated tissue factor protein samples were diluted in TBSA. Ten microliters were placed in a test tube with 50 μl of the factor IX_a/factor X reagent and 2 μl of a solution of purified factor VII, 30 units/ml. The tubes were warmed to 37°C and 100 μl 25mM $CaCl_2$ were added. Samples were incubated for 5 min. at 37°C prior to the addition of 50 μl chromogenic substrate S2222 containing the synthetic thrombin inhibitor I2581. The reaction was allowed to proceed for 10 min. and was stopped by the addition of 100 μl 50% glacial acetic acid solution. Absorbance was detected at 405 nm. A standard curve was

constructed using rabbit brain thromboplastin (commercially available from Sigma, St. Louis, MO. catalogue #T0263) arbitrarily assigning this reagent as having 100 tissue factor units/ml. Dilutions were made from 1:10 to 1:1000. Absorbance was plotted on the abscissa on semilog graph paper with dilution of standard plotted on the ordinate.

2. One stage assay for tissue factor activity.

100 μl haemophilic plasma were added to 10 μl of relipidated or lipid free tissue factor or TBSA as control in a siliconized glass tube to prevent non-specific activation through the contact phase of coagulation. The reactants were warmed to 37°C and 100 μl 25 mM $CaCl_2$ were added and clot formation timed. Hvatum, Y. and Prydz, H., Thromb. Diath. Haemorrh. 21, 217-222 (1969).

Tissue Preparation

Normal human saphenous veins and internal mammary arteries were obtained during coronary artery bypass surgery. Human atherosclerotic plaques were obtained from patients undergoing carotid endarterectomy surgery. Endarterectomy surgery consists of removal of an atherosclerotic plaque and some of the underlying smooth muscle. Additional normal tissue, including samples of normal organs and vessels prepared as described, for screening of tissue factor expression was obtained from a sacrificed Rhesus monkey.

The tissue samples were removed at surgery and immersed in freshly prepared 4% paraformaldehyde in 0.1M sodium phosphate (pH 7.4). The tissue was fixed at 4°C for 3 hrs. to overnight and then immersed in 15% sucrose phosphate buffered saline (PBS) for 2-4 hrs. at 4°C to act as a cryoprotectant. The tissue was then

embedded in an embedding medium for frozen tissue specimens ("OCT", Miles Laboratories) blocks and stored at -70°C. There was no loss of immunoreactivity or mRNA available for hybridization during this time. The tissue was sectioned at 10μm thickness using a cryostat, thaw-mounted onto poly-lysine coated microscope slides and immediately refrozen and stored at -70°C with dessicant.

In Situ Hybridization

In situ hybridizations were carried out as described previously (Rosenthal et al., EMBO J. 6:3641-3646 [1987]; Wilcox et al., Methods Enzymol. 124:510-533 [1986]). Prior to hybridization the sections were pretreated with paraformaldehyde (10 min.), proteinase K (1 μg/ml) (10 min.), and prehybridized for 1 to 2 hrs. in 50 μl of pre-hybridization buffer (0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 1x Denhardt's solution, 10% dextran sulfate and 10 mM dithiothreitol). The hybridizations were started by adding 300,000 CPMs of a tissue factor ^{35}S riboprobe in a small amount of pre-hybridization buffer. After hybridization the sections were washed with 2xSSC (2 x 10 min.) (1xSSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), treated with RNase (20 μg/ml, 30 min. room temperature), washed in 2xSSC (2 x 10 min.), followed by a high stringency wash in 0.1xSSC at 52°C for 2 hrs. All SSC solutions up to this point of the procedure contained 10 mM β-mercaptoethanol and 1 mM EDTA to help prevent non-specific binding of the probe. The tissue was then washed in 0.5xSSC without β-mercaptoethanol (2 x 10 min.) and dehydrated by immersion in graded alcohols containing 0.3M ammonium acetate. The sections were dried and coated with NTB2 nuclear emulsion (Kodak, Rochester, N.Y.) and exposed in the dark at 4°C for 4 to 8 weeks. After developing, the sections were counterstained with hematoxylin and eosin. The sections were developed at 15°C by treating the slides for 3 minutes in D19 developer) diluted 1:1 with water, Kodak), 20

seconds in water and 3 minutes in fixer. Slides were rinsed and counter stained.

A probe specific to human tissue factor (Fisher et al., Thrombosis Res. 48:89-99 [1987]) was labelled by transcription (Melton et al., Nucl. Acids Res. 12:7035-7056 [1984]) using [^{35}S]-labelled UTP (specific activity 1200 Ci/mmol, Amersham). The final specific activity of this probe was 300 Ci/mmol.

Immunocytochemistry

Immunocytochemistry was performed according to the manufacturer's direction using the Vectastain ABC alkaline phosphatase system (Vector, Inc., Burlingame, CA.). The final reaction product was stained with the alkaline phosphatase substrate kit I to give a final stain that appeared red. The anti-tissue factor protein antibody used was antibody RD010, described below.

An IgG fraction of the preimmune serum was used as a control for the tissue factor protein immunohistochemistry at the same IgG concentration as RD010. This was prepared by passing the preimmune serum over a protein A-Sepharose column. Frozen aliquots of all the antibody preparations were stored at -20°C until use.

Antibodies specific for human macrophages (HAM56, Gown, A.M. et al. Am. J. Pathol. 125:191-207 [1986]) or human endothelial cells (anti-Ulex Lectin, commercially available from Vector, Inc.) were also used to aid in cell identification.

Coagulation Assays

Procoagulant activity of tissue factor exposed in the plaque obtained during endarterectomy was measured using a modified

one-stage prothrombin time assay (Quick, A.J., Am. J. Physiol. 114:282-296 [1936]). Factor XII deficient plasma (100 μl) was incubated with or without a fragment of plaque tissue (21.2 $\pm$ 2.4 mg, mean $\pm$ S.D.) for 15 minutes at 37°C. The plaque was then removed and immediately 100 μl of cephalin reagent (a phospholipid) and 100 μl of 0.025M calcium chloride were added. The mixture was maintained at 37°C and the clotting time was noted visually.

In some experiments the plaque tissue was preincubated with a polyclonal antibody against recombinant tissue factor protein, for 15 minutes at 37°C in 50 mM tris buffer, pH 7.5, before addition to the plasma.

EXAMPLE 2

Production of Tissue Factor Protein Antagonists

The tissue factor protein antagonist, antibody RD010, was an affinity purified polyclonal antibody raised in rabbits. The RD010 was prepared by immunization of rabbits with tissue factor protein in Freund's adjuvant followed by boosters as required. Rabbits were immunized with recombinant human tissue factor protein produced in 293S cells as a fusion protein (see European Patent Application No. 88301190.0, filed February 12, 1988). The immune serum was purified by affinity chromatography on a recombinant human tissue factor-sepharose column. This antibody was shown to be monospecific on a western blot, to neutralize tissue factor activity, and immunoprecipitate tissue factor protein. This antibody was used at a dilution of 4.4 μg/ml for immunocytochemistry while 25-50 μg/ml was used for in vitro tissue factor inhibition studies.

Human monoclonal antibodies specific to tissue factor protein are generated using human B lymphocytes secreting antibody specific to tissue factor protein (see Human Hybridomas and Monoclonal Antibodies [eds. Engelman, E.G. et al., Plenum Press, 1985]). These B lymphocytes could be generated by in vitro immunizations. Tissue factor specific lymphocytes are transformed by Epstein-Barr virus or fused to immortal human lymphoblastoid, human myeloma, human plasmacytomas or other immortal cell lines. The immortalized line secretes tissue factor specific human IgG. Human recombinant molecule preparation may also be produced by the methods claimed in EP Publication No. 0125023, published November 14, 1984, which describes the combining of a variable region of a monoclonal antibody to the constant region of heavy or light chain of human antibodies.

EXAMPLE 3

Localization of Tissue Factor Protein

Normal Vessels

Normal human saphenous vein and internal mammary artery samples were examined for tissue factor protein biosynthesis. Endothelial cells were negative for tissue factor mRNA and protein (fig. 1a). Tissue factor positive cells were found dispersed in the tunica media and in the adventitia adjacent to the vessel (fig. 1a). The strongest labelling was seen over the adventitia where adventitial fibroblasts showed intense tissue factor protein staining (fib. 1b) and mRNA hybridization. Scattered cells in the tunica media contained tissue factor mRNA as determined by in situ analysis (fig. 1c). Immunochemical staining of the media, however, was unimpressive and fairly weak, but appeared to be cell associated and correlated well with the in situ results (fig. 1b). In general, more cells were found to be positive in the media by in

situ hybridization than could be detected by immunochemical staining. This may suggest reduced tissue translation or tissue factor secretion by these cells. Tissue factor positive cells in the media did not show typical smooth muscle cell morphology. The cytoplasm of these cells stained poorly with eosin, did not display a typical pancake shaped cytoplasm but rather appeared more cuboidal in shape and had small dense nuclei. Cells with this morphology typically do not stain with alpha smooth muscle actin antibodies (HHF35) and must be considered undefined.

The immunocytochemistry and in situ hybridization indicate that tissue factor protein is synthesized by smooth muscle cells in the tunica media and by fibroblasts in the adherent adventitia surrounding normal vessels. There was no evidence of tissue factor protein mRNA or protein localization in endothelial cells of any vessel studied. Previous cell culture work had suggested that induction of tissue factor protein synthesis by the endothelial cells represents a major procoagulant mechanism by which endothelial cells participate in homeostasis (Bevilacqua et al., Am. J. Pathol. 121:393-403 [1985]), and vascular smooth muscle cells have been shown to produce tissue factor protein at much higher levels (Maynard et al., J. Clin. Invest. 55:814-824 [1975]). Induction of endothelial tissue factor protein biosynthesis may be a normal mechanism by which the endothelium modifies homeostasis or alternatively be a response of the endothelium to infection and endotoxin stimulation.

Atherosclerotic Plaques

Human atherosclerotic plaques obtained from carotid endarterectomy surgery were examined for tissue factor mRNA and protein using the above techniques. Extensive mRNA hybridizations were seen in several regions of atherosclerotic plaque (fig. 2a).

Positive cells were found scattered throughout the fibrous cap, the base and shoulder region of the plaque as well as in the necrotic core adjacent to the cholesterol clefts (fig. 2c). The normal media underlying the endarterectomy specimens did not contain any tissue factor or mRNA positive cells. Ten plaques were screened and cells showing positive hybridization were seen in all of them. Immunohistochemistry and in situ hybridization labelled the same cells on serial sections.

The necrotic cores of the plaques were characterized by extensive tissue factor protein localization in the extracellular matrix particularly surrounding cholesterol clefts (fig. 2b). Additional protein staining was also seen in the macrophage-rich foam cell regions of at least one atherosclerotic plaque (fig. 3). Such foam cell-rich regions often lay underneath the fibrous cap and adjacent to the necrotic cores. Finally, as with the normal vessels, no tissue factor mRNA or protein was detected in surface or capillary endothelium. Similar staining patterns were seen on ten out of fourteen carotid endarterectomy specimens.

Atherosclerotic plaques were found to have significantly more tissue factor protein compared to normal saphenous veins, internal mammary artery, or regions of normal media underlying the plaque. Tissue factor protein mRNA was found in both mesenchymal like intimal cells in the atherosclerotic intima as well as in macrophages and cells adjacent to the cholesterol clefts which appear to be macrophages as well. Immunocytochemistry indicated that there is a considerable amount of tissue factor protein trapped in the extracellular matrix of the necrotic core of the atherosclerotic plaque. This is not cell associated but is locally synthesized since cells adjacent to these regions contain tissue factor mRNA. Tissue factor protein found in the necrotic core may

be shed from the cell surface of the synthetic cells and subsequently trapped in the surrounding lipid matrix. Alternatively, the tissue factor protein in this region may originate from cells that have died and left tissue factor rich membranes behind.

The immunostaining of macrophage foam cells suggests that in these cells tissue factor is intracellular as well as possibly cell surface associated. To what extent such stores of tissue factor are macrophage-derived or whether this protein originates from phagocytosis of surrounding necrotic core debris is not clear. Levy et al., J. Clin. Invest. 67:1614-1622 (1981) have shown that certain lipoprotein fractions can induce procoagulant activity originating from monocytes/macrophages. The production of tissue factor by macrophages has been demonstrated by other investigators (Tipping et al., Am. J. Pathol. 131:206-212 [1988]; Levy et al., supra). In addition, we have shown macrophages with tissue factor mRNA. Tissue factor represents the final common pathway for both the intrinsic and extrinsic pathways of coagulation (Nemerson, Y. Blood 71:1-8 [1988]). It is a highly thrombogenic peptide and requires only phospholipid and factor VII/VIIa to activate factor X directly and indirectly via factor IX activation, leading to the generation of thrombin. Factor VII is normally present in blood but requires binding to tissue factor for activation of factor X (Nemerson, Y., supra). Since there is no in vivo coagulation in the absence of vascular damage it is reasonable to assume that tissue factor would not normally be exposed to blood (Nemerson, supra; Spicer et al., PNAS [USA] 84:5148-5152 [1987]). This is consistent with our findings since normal vessel endothelial cells in direct contact with the blood do not synthesize or store tissue factor. Since tissue factor is found in scattered smooth muscle cells in the tunica media and adventitial cells adherent to the

vessel, vessel wall rupture into these areas would be required to expose the blood to significant stores of procoagulant tissue factor activity. Zaugg et al., J. Clin. Chem. and Clin. Biochem. 18:545 (1980) have shown that damaged human aorta exposes factor VII dependent procoagulant activity in support of this hypothesis.

EXAMPLE 4

Tissue Factor Coagulation Assays

The thrombogenicity of tissue factor protein detected by immunocytochemistry was assessed using snap-frozen unfixed tissue taken from carotid endarterectomy surgery in coagulation assay (fig. 4). Coagulation time without addition of plaque tissue was in excess of 360 seconds. Preincubation of the tissue in factor XII deficient plasma accelerated the coagulation time over that with serum alone. This acceleration of coagulation could be inhibited by the addition of increasing amounts of tissue factor neutralizing antibody, RD010. These results indicate that the tissue factor protein in the endarterectomy material was capable of participating in the initiation of coagulation. Multiple plaques were screened for tissue factor protein activity and all showed similar results.

Advanced human atherosclerosis is characterized by intimal smooth muscle cell proliferation accompanied by accumulation of fats and inflammatory cells including macrophages and T cells within the atherosclerotic plaque (Ross, N. Eng. J. Med. 314:488-500 [1986]; Gown et al., Am. J. Pathol. 125:191-207 [1986]; Jonasson, L. et al., Arteriosclerosis 6:131-138 [1986]). Thrombosis is commonly the critical event which converts an asymptomatic atherosclerotic plaque into a symptomatic one (Falk, Br. Heart J. 50:127-134 [1983]; Sherman et al., N. Eng. J. Med.

315:913-919 [1986]) whereas non-diseased arteries hardly ever become thrombosed. Plaque rupture is likely to be the integral event that precipitates clot formation (Forrester et al., Circulation 75:505-513 [1987]). An occlusive mural thrombus accompanies most cases of acute myocardial infarctions (Buja, L.M. et al., Am. J. Cardiol. 47:343-356 [1981]). Plaque rupture or cracking is normally found to underlie such thrombi, and in many cases the thrombus is seen to extend into the region of the necrotic core of the plaque extruding through such cracks. This is true of both the coronary (Falk, Br. Heart J. 50:127-134 [1983]; Chapman,I., Arch. Pathol. 80:256-261 [1965]; Drury, J. Path. Bact. 67:207-215 [1954]) and cerebral arteries (Constantinides, Arch. Pathol. 83;422-428 [1967]). The source of the thrombogenicity of the plaque has not previously been determined but these results indicate that a thrombus results when blood components come into contact with the tissue factor protein present within the plaque. The results show that there is a) significant synthesis of tissue factor protein in atherosclerotic plaques; b) that tissue factor protein accumulates in the necrotic core and is found in foam cell rich regions of the plaque; and c) that there is in the plaque procoagulent activity due to tissue factor protein as identified by in vitro coagulation assays that is significantly reduced by tissue factor protein antagonists. The overproduction and trapping of tissue factor protein in the atherosclerotic plaque may play a role in thrombosis and reocclusion of an atherosclerotic vessel following thrombolytic therapy.

EXAMPLE 5

Test for Coronary Artery Thrombosis

Mongrel dogs weighing approximately 20-25 kg are anesthetized with a slow intravenous injection of sodium

pentobarbital, incubated and placed on an artificial ventilator. A left thoracotomy is performed in the 5th-6th intercostal space, and an arterial catheter is placed in the internal mammary artery for blood pressure monitoring. Procainamide (1.5 g injected intramuscularly in 2-3 sites) is then provided, the pericardium is opened, and a pericardial cradle is prepared. The left anterior descending coronary artery is dissected out from the epicardium, side branches are ligated, and a 2.5 cm segment is isolated. An electromagnetic flow probe (Carolina Medical Electronics FM501, King, NC) is placed on the most proximal portion of the segment and intravenous lidocaine (15 mg bolus followed by a constant infusion at 1 mg/min) is infused. A control left coronary angiogram is performed by injecting approximately 2 ml of Renografin 76, by hand, through a modified Judkin's 7 French catheter inserted from a carotid artery. 1 ml of blood is then removed and kept in a syringe for later use in forming the thrombus, and heparin (5000 U intravenous bolus) is administered. Additional 1000 U boluses of heparin are administered at hourly intervals. A permanent 2 mm wide constrictor is placed near the distal end of the segment and adjusted so as to reduce coronary artery blood flow to approximately 40 $\pm$ 10% of control.

High resolution post-mortem angiograms in selected animals show that a constriction, so placed, decreases the luminal diameter by more than 90%. The 1 cm of coronary artery just proximal to the constriction is then emptied of blood and isolated between temporary silk snares. Intimal damage is induced by grasping the segment with forceps, and then the segment is flushed by releasing the proximal snare and injection of saline retrograde through a cannulated side branch. The segment is then reisolated and 0.2 ml of thrombin (Parke-Davis topical thrombin, 1000 U/ml, Morris Plains, NJ) is introduced. 0.1 ml of the stored blood is injected

into this isolated segment. After approximately 5 minutes, first the proximal and then the distal ties are released and the side branch catheter is removed. During this procedure, the permanent constrictor remained in place.

Approximately 30 minutes after injecting the thrombin and blood, and after a repeated angiogram confirms the presence of a complete coronary artery occlusion, slow intravenous injections of tissue factor protein antagonists, acetylsalicylic acid (35 mg/kg) or dipyridamole (0.6 mg/kg) are administered. Approximately 10 minutes later, a 30-minute infusion of rt-PA (15 μg/kg/min for the two chain form or 30 μg/kg/min for the single chain form) is initiated.

If partial coronary artery reperfusion does not occur within the 30-minute infusion period, rt-PA infusion is continued for an additional 30 minutes. The blood flow in the affected vessel is monitored continuously. An angiogram is immediately performed after restoration of blood flow. The reperfusion time is taken as the number of minutes from the beginning of the rt-PA infusion until reperfusion is documented by the flow meter and is confirmed by the repeat angiogram showing complete antegrade filling of the artery with rapid clearance of the dye (less than 4 cardiac cycles). After reperfusion is obtained, blood flow is monitored for evidence of reocclusion, with a final confirmation again being obtained by angiography, using the same criteria as are used for establishing reperfusion. The reocclusion time is taken as the interval between documented reperfusion and reocclusion. The above described animal model closely simulates the response to thrombolytic therapy by human patients having acute myocardial infarction.

Bleeding times are performed before and 30 min after injections of the tissue factor protein antagonists with a spring-loaded blade device (Simplate, General Diagnostic, Morris Plains, NJ or Surgicutt Int. Technidyne Corp., Edison, NJ), applied to a shaved foreleg. Venous blood samples for determination of the levels of fibrinogen, activated partial thromboplastin time, ADP-induced platelet aggregation and ^{125}I-7E3 binding are collected into 0.01 M citrate containing 150 KIU/ml aprotinin. (Sigma, St. Louis, MO.) Platelet counts are performed on blood drawn into EDTA using an automated particle counter (Coulter, Hialeah, FL).

EXAMPLE 6

Comparison of Thrombolytic Potency and Effect on Reocclusion of Bolus Injections of rt-PA and rt-PA plus Tissue Factor Protein Antagonists

The thrombolytic potency and effect on reocclusion of bolus injections of rt-PA alone is compared to that of combined injections of rt-PA and tissue factor protein antagonist using the animal model of Example 5.

Bolus injections of 450 μg/kg of rt-PA at 15 min intervals is given (with high grade [over 90%] superimposed stenosis).

Injection of about 0.01 to 5.0 mg/kg of tissue factor protein antagonist is followed 10 min later by a single bolus injection of 450 μg/kg of rt-PA in dogs to test for reperfusion within 5-10 min without reocclusion during an observation period of 2 hours.

5/4/004

Claims:

1. A method of treating a patient having a myocardial infarction comprising administration of a therapeutically effective amount of tissue factor protein antagonist to prevent reocclusion and a thrombolytic agent in a therapeutically effective amount to dissolve a fibrin-platelet clot.

2. A method of treating a patient having angioplasty comprising administration of a therapeutically effective amount of tissue factor protein antagonist to prevent reocclusion and a thrombolytic agent in a therapeutically effective amount to dissolve a fibrin-platelet clot.

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3. The method of claim 1 or 2 wherein administration to the patient is by intravenous infusion.

4. The method of claim 1 or 2 wherein administration to the patient is by bolus.

5. The method of claim 4 wherein the bolus is intravenously injected.

6. The method of claim 4 wherein the patient is administered a first bolus containing the tissue factor protein antagonist and subsequently administered a second bolus containing the thrombolytic agent.

7. The method of claim 4 wherein the patient is administered a first bolus containing the thrombolytic agent and subsequently administered a second bolus containing the tissue factor protein antagonist.

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8. The method of any one of claims 1 - 4 wherein tissue factor protein antagonist is an antibody.

9. The method of claim 8 wherein the antibody is a polyclonal antibody.

10. The method of claim 8 wherein the antibody is a monoclonal antibody.

11. The method of any one of claims 1-4 wherein said thrombolytic agent is selected from the group consisting of streptokinase, urokinase, prourokinase and tissue-type plasminogen activator.

12. The method of claim 11 wherein said thrombolytic agent is tissue-type plasminogen activator.

13. The method of any one of claims 1-3 or 7 wherein the tissue factor protein antagonist is given at a dose of about in the range of 0.01 - 4.0 mg per kg of patient weight and the thrombolytic agent is given at a dose of about in the range of 0.01 - 2.5 mg per kg of patient weight.

14. A pharmaceutical composition for the treatment of myocardial infarction comprising therapeutically effective amounts of a tissue factor protein antagonist and thrombolytic agent.

15. The pharmaceutical composition of claim 14 wherein the tissue factor protein antagonist is an antibody.

16. The pharmaceutical composition of claim 14 wherein the thrombolytic agent is selected from the group consisting of streptokinase, urokinase, prourokinase and tissue-type plasminogen activator.

Abstract of the Disclosure

The present invention relates to a method and therapeutic composition for the treatment of myocardial infarction comprising administration of a tissue factor protein antagonist and a thrombolytic agent.

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APPLICANT(S) DONOGH P. O'BRIEN, SAN BRUNO, CA; GORDON A. VEHAR, SAN CARLOS, CA.

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TITLE
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METHOD AND THERAPEUTIC COMPOSITIONS FOR THE TREATMENT OF BLEEDING DISORDERS

This invention relates to the treatment of bleeding disorders. In particular, this invention relates to the use of tissue factor protein to effect haemostasis in certain clinical conditions and particularly in animals lacking certain coagulation proteins. Factor VIII and factor IX deficiencies are two examples.

Bleeding is one of the most serious and significant manifestations of disease. It may occur from a local site or may be generalized. Bleeding associated with a local lesion may be superimposed on either a normal or a defective haemostatic mechanism. Normal haemostasis comprises mechanisms operative immediately following an injury and those acting over a longer period. Primary haemostasis consists principally of two components: vasoconstriction and platelet plug formation. The maintenance mechanism consists of the fibrin clot produced by the coagulation system. Platelet plug formation is especially important in capillary haemostasis, while vasoconstriction and fibrin clot formation is more important in larger vessel haemostasis. In the microcirculation haemostasis consists of vasoconstriction and platelet plug formation. Platelet plug formation may be divided into several stages: adhesion of platelets to subendothelial surfaces exposed by trauma; platelet

activation release reaction; platelet aggregation, which results in the sequestration of additional platelets at the site, and the binding of fibrinogen and the coagulation proteins to the platelet surface which includes thrombin formation; and, fusion which is the coalescence of fibrin and fused platelets to form a stable haemostatic plug.

Blood coagulation performs two functions; the production of thrombin which induces platelet aggregation and the formation of fibrin which renders the platelet plug stable. A number of discrete proenzymes and procofactors, referred to as "coagulation factors", participate in the coagulation process. The process consists of several stages and ends with fibrin formation. Fibrinogen is converted to fibrin by the action of thrombin. Thrombin is formed by the proteolytic cleavage of a proenzyme, prothrombin. This proteolysis is effected by activated factor X (referred to as factor X_a) which binds to the surface of activated platelets and in the presence of Va and ionic calcium cleaves prothrombin.

Activation of factor X may occur by either of two separate pathways, the extrinsic or the intrinsic (Figure 1). The intrinsic cascade consists of a series of reactions wherein a protein precursor is cleaved to form an active protease. At each step, the newly formed protease will catalyze the activation of the precursor protease at the subsequent step of the cascade. A deficiency of any of the proteins in the pathway blocks the activation process at that step, thereby preventing clot formation and typically gives rise to a tendency to hemorrhage. Deficiencies of factor VIII or factor IX, for example, cause the severe bleeding syndromes haemophilia A and B, respectively. In the extrinsic pathway of blood coagulation, tissue factor, also referred to as tissue thromboplastin, is released from damaged cells and activates factor X in the presence of factor VII and calcium. Although activation of factor X was originally believed to be the only reaction catalyzed by tissue factor and factor VII, it is now known that an amplification loop exists between factor X,

factor VII, and factor IX (Osterud, B., and S.I. Rapaport, Proc. Natl. Acad. Sci. USA 74:5260-5264, 1977; Zur, M. et al., Blood 52: 198, 1978). Each of the serine proteases in this scheme is capable of converting by proteolysis the other two into the activated form, thereby amplifying the signal at this stage in the coagulation process (Figure 2). It is now believed that the extrinsic pathway may in fact be the major physiological pathway of normal blood coagulation (Haemostasis 13:150-155 1983). Since tissue factor is not normally found in the blood, the system does not continuously clot; the trigger for coagulation would therefore be the release of tissue factor from damaged tissue.

Tissue factor is an integral membrane glycoprotein which, as discussed above, can trigger blood coagulation via the extrinsic pathway. Bach, R. et al., J. Biol Chem. 256(16), 8324-8331 (1981). Tissue factor consists of a protein component (previously referred to as tissue factor apoprotein-III) and a phospholipid. Osterud, B. and Rapaport, S.I., PNAS 74, 5260-5264 (1977). The complex has been found on the membranes of monocytes and different cells of the blood vessel wall. Osterud, B., Scand. J. Haematol. 32, 337-345 (1984). Tissue factor from various organs and species has been reported to have a relative molecular mass of 42,000 to 53,000. Human tissue thromboplastin has been described as consisting of a tissue factor protein inserted into phospholipid bilayer in an optimal ratio of tissue factor protein:phospholipid of approximately 1:80. Lyberg, T. and Prydz, H., Nouv. Rev. Fr. Hematol 25(5), 291-293 (1983). Purification of tissue factor has been reported from various tissues such as,: human brain (Guha, A. et al. PNAS 83, 299-302 [1986] and Broze,G.H. et al., J.Biol.Chem. 260[20], 10917-10920 [1985]); bovine brain (Bach, R. et al., J. Biol. Chem. 256, 8324-8331 [1981]); human placenta (Bom, V.J.J. et al., Thrombosis Res. 42:635-643 [1986]; and, Andoh, K. et al., Thrombosis Res. 43:275-286 [1986]); ovine brain (Carlsen, E. et al., Thromb. Haemostas. 48[3], 315-319 [1982]); and, lung (Glas, P. and Astrup, T., Am. J. Physiol. 219, 1140-1146 [1970]. It has been shown that bovine and human tissue thromboplastin are

identical in size and function. See for example Broze, G.H. et al., J. Biol. Chem. 260(20), 10917-10920 (1985). It is widely accepted that while there are differences in structure of tissue factor protein between species there are no functional differences as measured by in vitro coagulation assays. Guha et al. supra. Furthermore, tissue factor isolated from various tissues of an animal, e.g. dog brain, lung, arteries and vein was similar in certain respects such as, extinction coefficient, content of nitrogen and phosphorous and optimum phospholipid to lipid ratio but differed slightly in molecular size, amino acid content, reactivity with antibody and plasma half life. Gonmori, H. and Takeda, Y., J. Physiol. 229(3), 618-626 (1975). All of the tissue factors from the various dog organs showed clotting activity in the presence of lipid. Id. It is widely accepted that in order to demonstrate biological activity, tissue factor must be associated with phospholipids. Pitlick, F.A., and Nemerson, Y., Biochemistry 9, 5105-5111 (1970) and Bach,R. et al. supra. at 8324. It has been shown that the removal of the phospholipid component of tissue factor, for example by use of a phospholipase, results in a loss of its biological activity. Nemerson, Y., J.C.I. 47, 72-80 (1968). Relipidation can restore in vitro tissue factor activity. Pitlick, F.A. and Nemerson, Y. Biochemistry 9, 5105-5113 (1970) and Freyssinet, J.M. et al., Thrombosis and Haemostasis 55, 112-118 [1986].

Infusion of tissue factor has long been believed to compromise normal haemostasis. In 1834 the French physiologist de Blainville first established that tissue factor contributed directly to blood coagulation. de Blainville, H. Gazette Medicale Paris, Series 2, 524 (1834). de Blainville also observed that intravenous infusion of a brain tissue suspension caused immediate death which he observed was correlated with a hypercoagulative state giving rise to extensively disseminated blood clots found on autopsy. It is now well accepted that intravenous infusion of tissue thromboplastin induces intravascular coagulation and may cause death in various animals. (Dogs: Lewis, J. and Szeto I.F., J. Lab. Clin. Med. 60, 261-273

(1962); rabbits: Fedder, G. et al., Thromb. Diath. Haemorrh. 27, 365-376 (1972); rats: Giercksky, K.E. et al., Scand. J. Haematol. 17, 305-311 (1976); and, sheep: Carlsen,E. et al., Thromb. Haemostas. 48, 315-319 [1982]).

In addition to intravascular coagulation or a hypercoagulative state resulting from the exogenous administration of tissue factor, it has been suggested that the intravascular release of tissue thromboplastin may initiate disseminated intravascular coagulation (DIC). Prentice, C.R., Clin. Haematol. 14(2), 413-442 (1985). DIC may arise in various conditions such as shock, septicaemia, cardiac arrest, extensive trauma, bites of poisonous snakes, acute liver disease, major surgery, burns, septic abortion, heat stroke, disseminated malignancy, pancreatic and ovarian carcinoma, promyelocytic leukemia, myocardial infarction, neoplasms, systemic lupus erythematosus, renal disease and eclampsia. Present treatment of DIC includes transfusion of blood and fresh frozen plasma; infusion of heparin; and removal of formed thrombi. The foregoing clinical syndromes suggest that endogenous release of tissue factor can result in severe clinical complications. Andoh, K. et al., Thromb. Res. 43, 275-286 (1986). Efforts were made to overcome the thrombotic effect of tissue thromboplastin using the enzyme thromboplastinase. Gollub, S. et al., Thromb. Diath. Haemorh. 7, 470-479 (1962). Thromboplastinase is a phospholipase and would presumably cleave the phospholipid portion of tissue factor. Id.

Congenital disorders of coagulation characteristically involve a single coagulation protein. Haemophilia is a bleeding disorder due to inherited deficiency of a coagulation factor, e.g. the procoagulant activity of factor VIII. The basis for therapy of bleeding episodes is transfusion of material containing the missing coagulation protein, e.g. infusion of factor VIII procoagulant activity which temporarily corrects the specific defect of haemophilia A.

Von Willebrand's disease is another bleeding disorder characterized by a prolonged bleeding time in association with an abnormality or deficiency in the von Willebrand protein. Treatment is by infusion of normal plasma or by a composition rich in von Willebrand protein. Congenital deficiencies of each of the other coagulation factors occur and may be associated with a haemorrhagic tendency. The present therapies for the deficiencies are: factor IX deficiency is treated using concentrates containing factor IX ; infusions of plasma are given for a factor XI deficiency; and plasma infusion is given for a factor XIII deficiency.

Acquired coagulation disorders arise in individuals without previous history of bleeding as a result of a disease process. Inhibitors to blood coagulation factors may occur in multitransfused individuals. Acquired coagulation factor deficiencies with unknown etiology also give rise to haemostatic problems. DIC describes a profound breakdown of the haemostasis mechanism.

This invention is based in part on the novel and unexpected observation that infusion into rabbits lacking coagulation factors of tissue factor protein, that is the protein portion of tissue factor lacking the naturally occurring phospholipid, which was previously referred to as tissue factor apoprotein III and previously believed to be inactive, corrected the haemostatic deficiency. Tissue factor protein was for the first time found to correct the bleeding diathesis, i.e. a tendency toward hemorrhage, associated with factor VIII deficiency in vivo. Furthermore, infusion of tissue factor protein would be expected to be ineffective in light of the papers which describe tissue factor as having an absolute requirement for phospholipid. In contrast to the work of de Blainville and subsequent researchers over the next one hundred and fifty-two (152) years, tissue factor protein was also found to be nontoxic to rabbits when infused intravenously.

An object of the present invention is to provide a coagulation

inducing therapeutic composition for various chronic bleeding disorders, characterized by a tendency toward hemorrhage, both inherited and acquired. Examples of such chronic bleeding disorders are deficiencies of factors VIII, IX, or XI. Examples of acquired disorders include: acquired inhibitors to blood coagulation factors e.g. factor VIII, von Willebrand factor, factors IX, V, XI, XII and XIII; haemostatic disorder as a consequence of liver disease which includes decreased synthesis of coagulation factors and DIC; bleeding tendency associated with acute and chronic renal disease which includes coagulation factor deficiencies and DIC; haemostasis after trauma or surgery; patients with disseminated malignancy which manifests in DIC with increases in factors VIII, von Willebrand factor and fibrinogen; and haemostasis during cardiopulmonary surgery and massive blood transfusion. Another object of this invention is to provide a method of treatment of such chronic bleeding disorders.

A further object of this invention is to provide a coagulation inducing therapeutic composition for acute bleeding problems in normal patients and in those with chronic bleeding disorders. Another object of this invention is to provide a method of treatment for such acute bleeding problems.

Yet another object of this invention is to provide an anticoagulant therapeutic, that is an antagonist to tissue factor protein, to neutralize the thrombotic effects of endogenous release of tissue thromboplastin which may result in a hypercoagulative state. Particularly, such an anticoagulant, that is an antagonist to tissue factor protein, would neutralize the hypercoagulant effects of endogenously released tissue thromboplastin by inactivating tissue factor protein. Such a tissue factor protein antagonist can be an antibody or other protein that specifically inactivates the protein component.

Summary of the Invention

This invention has been demonstrated by the novel discovery that administration of tissue factor protein, that is tissue thromboplastin or tissue factor lacking the phospholipid normally associated with the naturally occurring protein, to haemostatic deficient animals corrected the deficiency. Tissue factor protein until the unexpected observations of this invention, was considered inactive in the absence of the phospholipid component. Tissue factor protein was for the first time found to be nontoxic when administered intravenously. Accordingly, in one aspect the invention is directed to administration of a pharmaceutical composition comprising tissue factor protein as a coagulant in patients with bleeding disorders. In another aspect the invention is directed to a method of treatment of chronic bleeding disorders. Yet another aspect is a method of treatment of acute bleeding incidents in patients having chronic bleeding disorders. A further aspect of this invention is directed to an anticoagulant to neutralize the coagulant effects of endogenously released tissue thromboplastin by inactivating tissue factor protein.

Brief Description of the Drawings

Figure 1. Diagram showing activation of blood coagulation via intrinsic pathway.

Figure 2. Diagram showing amplification of coagulation signal via extrinsic pathway.

Detailed Description

As used herein, "tissue factor protein" refers to a protein capable of correcting various bleeding disorders, particularly those associated with deficiencies in coagulation factors. Tissue factor protein is distinct from tissue factor or tissue thromboplastin in that it lacks the naturally occurring lipid portion of the molecule. Infusion of tissue factor protein does not result in disseminated intravascular coagulation.

The term "tissue factor protein antagonists" as used herein refers to substances which may function in two ways. First, tissue factor protein antagonists will bind to tissue factor protein with sufficient affinity and specificity to neutralize tissue factor protein such that it cannot bind to factor VII or VII_a nor effect the proteolysis of factors IX or X when in complex with factor VII or VII_a. Alternatively, tissue factor protein antagonists will inactivate tissue factor protein or the tissue factor/factor VII_a complex by cleavage, e.g. a specific protease. Antagonists are useful, either alone or together, in the therapy of various coagulation disorders as evidenced by altered plasma fibrinogen levels as described herein e.g. DIC occurring during severe infections and septicemias, after surgery or trauma, instead of or in combination with other anticoagulants such as heparin.

An example of an antagonist which will neutralize tissue factor protein is a neutralizing antibody to tissue factor protein. Tissue factor protein neutralizing antibodies are readily raised in animals such as rabbits or mice by immunization with tissue factor protein in Freund's adjuvant followed by boosters as required. Immunized mice are particularly useful for providing sources of B cells for the manufacture of hybridomas, which in turn are cultured to produce large quantities of inexpensive anti-tissue factor protein monoclonal antibodies. Such tissue factor protein monoclonal antibodies have been prepared by Carson, S.D. et al., Blood 66(1), 152-156 (1985).

Tissue factor is released from damaged cells and activates factors IX and X in the presence of factor VII or VII_a and calcium. (See Figure 2) The activation of factor X by the extrinsic pathway of coagulation has an absolute requirement for tissue factor. Silverberg, S.A., et al., J. Biol. Chem. 252, 8481-8488 (1977). Until the discovery of this invention, it was thought that the lipid component of tissue factor was essential for optimal tissue factor

activity in the catalysis of factor X or factor IX by factor VII or VII_a. This invention encompasses the treatment of various acute and chronic bleeding disorders by bypassing those deficiencies through the administration of tissue factor protein. More particularly this invention is applicable to those bleeding disorders arising in animals deficient in various coagulation factors.

Tissue thromboplastin or tissue factor consists of a glycoprotein component (previously referred to as tissue factor apoprotein III) which has been purified to apparent homogeneity (Bjorklid, E. et al., Biochem. Biophys. Res. Commun. 55, 969-976 [1973]) and a phospholipid fraction. Numerous reports have described the purification of tissue factor from many types of tissue such as brain, lung and placenta. Sheep, cow, rabbit, dog and human have been a source of tissue factor. The first step in the chemical purification has been to dissociate tissue factor from its native lipid using, for example, extraction with organic solvents. Examples of such organic solvents include pyridine, heptane-butanol mixture or ethanol. Tissue factor protein has been purified by chemical means. Examples of such chemical means are: treatment with detergents, such as deoxycholate or Triton X-100; gel filtration and preparative polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate; concanavalin A bound to a Sepharose column; and, affinity columns using antibodies to the tissue factor protein or selective adsorption to factor VII. Included within the scope of tissue factor protein is tissue factor protein from recombinant or synthetic sources. Also included are dimers of tissue factor protein and tissue factor protein variants having amino acid substitutions and/or deletions and/or additions, organic and inorganic salts and covalently modified derivatives of tissue factor protein. Tissue factor protein produced by recombinant means may include a naturally occurring pro-form as well as a prepro-form of tissue factor protein.

For use in this invention tissue factor protein or tissue factor protein antagonists may be formulated into an injectable

preparation. Parenteral formulations are suitable for use in the invention, preferably for intravenous administration. These formulations contain therapeutically effective amounts of tissue factor protein, are either sterile liquid solutions, liquid suspensions or lyophilized versions and optionally contain stabilizers or excipients. Typically, lyophilized compositions are reconstituted with suitable diluents, e.g. sterile water for injection, sterile saline and the like where the biological activity is sufficient to induce haemostatic coagulation as observed in a rabbit infusion study.

Alternatively, for use in this invention tissue factor protein can be formulated into a preparation for absorption through the gastrointestinal tract. Such a preparation is suitable for use in the invention for oral administration. Such oral preparations contain therapeutically effective amounts of tissue factor protein, a lipophilic vehicle and a gastrointestinal tract absorption enhancing agent. Suitable lipophilic vehicles include mineral oil, triglycerides, esterified glycols, polyglycols with hydrophobic alkyl side chains, and sterols. Examples of an absorption enhancer include hydroxyaryl or hydroxyaralkyl acids or their salts, esters or amides. Other compounds with similar properties include salicylic acid derivatives, amines of 1,3 dicarbonyl compounds and enamino acids, and their salts, amides and esters.

Tissue factor protein may be administered by injection intravascularly or by oral administration at a dosage sufficient to correct a bleeding disorder, for example, replacement therapy in the face of a factor VIII deficiency. Tissue factor protein may be administered at a dosage sufficient to correct an acute bleeding incident in the face of a coagulation factor deficiency. Therapeutic dosage of tissue factor protein is in the range of about from 10 U/kg to 100 U/kg. A preferred therapeutic dosage of tissue factor protein is in the range of about 25 U/kg to 75 U/kg. A most preferred therapeutic dosage of tissue factor protein is in the range of about

protein would be in the range of from about 1750 U (70 x 25 U/kg) to 5250 U (70 x 75 U/kg). A most preferred therapeutic dosage of tissue factor protein is in the range of about 35 U/kg to 65 U/kg. In the absence of an international standard of tissue factor activity we have established a tissue factor standard. A unit of tissue factor activity is that amount of tissue factor protein in 10 μl of tissue thromboplastin (commercially available from Sigma, St. Louis, MO) as measured by the chromogenic assay. See description of chromogenic assay below. The dose will be dependent upon various therapeutic variables including the animal species to be treated, the route of administration, the properties of the tissue factor protein employed, e.g. its activity and biological half life, the concentration of tissue factor protein in the formulation, the patient's plasma volume, the clinical status of the patient e.g. the particular bleeding disorder, and such other parameters as would be considered by the ordinarily skilled physician.

Tissue factor protein antagonist may be administered by injection intravascularly at a dosage sufficient to correct a bleeding disorder, e.g. DIC. Antagonists may be administered at a dosage sufficient to correct such a bleeding disorder. The dose will be dependent on various therapeutic variables known to the ordinarily skilled artisan.

Tissue factor protein also is suitably formulated into a topical preparation for local therapy for minor bleeding occurring from an accessible site in conjunction with a cold application and gentle pressure. Such a preparation for local therapy includes a therapeutically effective concentration of tissue factor protein in a dermatological vehicle. The amount of tissue factor protein to be administered and the tissue factor protein concentration in the topical formulation, will depend on the vehicle selected, the clinical condition, the species of tissue factor protein used and the stability of tissue factor protein in the formulation.

The tissue factor protein or antagonist of this invention preferably is formulated and administered as a sterile solution although it is within the scope of this invention to utilize lyophilized tissue factor preparations. Sterile solutions are prepared by sterile filtration of tissue factor protein or by other methods known per se in the art The solutions are then lyophilized or filled into pharmaceutical dosage containers. The pH of the solution should be in the range of pH 3.0 to 9.5, preferably pH 5.0 to 7.5. The tissue factor protein should be in a solution having a suitable pharmaceutically acceptable buffer such as phosphate, tris (hydroxymethyl) aminomethane-HCl or citrate and the like. Buffer concentrations should be in the range of 1 to 100 mM. The solution of tissue factor protein may also contain a salt, such as sodium chloride or potassium chloride in concentration of 50 to 750 mM. An effective amount of a stabilizing agent such as an albumin, a globulin, a gelatin, may also be included and may be added to a solution containing tissue factor protein. An effective amount of detergent such as sodium deoxycholate or Triton X-100 or sodium dodecyl sulfate (SDS) may be added.

Tissue factor protein or antagonist preferably is placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper piercable by a hypodermic injection needle.

Systemic administration of tissue factor protein may be made daily or several times a week in the case of replacement therapy for a coagulation factor deficiency. Administration is typically by intravenous injection. Administration may also be intranasal or by other nonparenteral routes. Tissue factor protein may also be administered via microspheres, liposomes or other microparticulate delivery systems placed in certain tissues including blood.

Example 1

General Materials and Methods

Mature bovine brains were obtained from Pel-Freeze, Rogers, Ar., and stored at -20°. Triton X-100 and α-D-methylglucoside were from Calbiochem, San Diego, CA. Concanavalin A-Sepharose resin (referred to as Con A Sepharose in Table 1) was from Pharmacia and Ultrogel AcA 44 from LKB, Gaithersburg, MD. All chemicals and reagents for preparative and analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories, Richmond, CA. Factor IX_a/Factor X reagent and S2222/I2581 were obtained from Helena Laboratories (Kabi Coatest kit, Helena Laboratories, Beaumont, CA., Catalogue No. 5293). YM 10 ultrafiltration membranes were from Amicon. Factor VII was purified from bovine plasma. (Broze, G. and Majerus, P., J. Biol. Chem. 255(4): 1242-1247 [1980]). Factor VIII deficient and normal pooled citrated plasma were from George King Biomedicals, Overland Park, Kansas. Crude phosphotidylcholine (lecithin granules from soya bean) were obtained from Sigma, St. Louis, MO. All other chemicals were of reagent grade or better.

Acetone Delipidation of Bovine Brains

Two mature bovine brains were thawed at room temperature and rinsed free of clotted blood with distilled water. The tissue was then homogenized into ice cold acetone to a volume of 10 ml acetone per gram wet weight of bovine brain using an Ultra-Turrex tissue homogenizer. The homogenate was extracted at 4°C for 30 min. and then filtered through Whatman No. 1 filter paper on an evacuated flask. The tissue slurry was resuspended in the original volume of ice cold acetone, extracted and filtered for six times. The final filter cake was dried under a stream of nitrogen and stored at-20°C.

Triton X-100 Solubilization of Tissue Factor

Acetone brain powders (145 g) were homogenized in 0.05 M Tris/0.1 M NaCl, pH 7.5 (TBS) to a final volume of 20 ml buffer/g acetone brain powder. The homogenate was extracted at 4°C for 1 hr. and subsequently centrifuged at 10,000 x g for 1 hr. at 4°C. The supernatant was discarded and the pellet re-homogenized into three (3) liters TBS/0.1% Triton X-100. The material was extracted and centrifuged as before. The pellet thus obtained was then homogenized into three (3) liters TBS/2% Triton X-100 to solubilize tissue factor. The homogenate was extracted for 16 hrs. at 4°C and then centrifuged as before.

Concanavalin A-Sepharose Affinity Column

The supernatant from the 2% Triton X-100 extraction was made 1 mM in $CaCl_2$ and $MgCl_2$ and batch adsorbed with 100 ml Concanavalin-A Sepharose resin for 16 hrs at 4°C. Following adsorption, the Sepharose resin was poured into a 3 x 20 cm column and washed with 500 ml TBS 0.05% Triton X-100 at a flow rate of 2 ml/min. Absorbance was monitored at 280 nM. When no further protein washed from the column, the Sepharose was eluted isocratically with a buffer comprising 100 mg/ml α-D methylglucoside in TBS/0.05% Triton X-100. Ten milliliter fractions were collected at a flow rate of 2 ml/min. Fractions were relipidated and assayed for tissue factor activity. Tissue factor protein was eluted in approximately four (4) column volumes of eluant. The eluate was concentrated in an Amicon concentration cell using a YM 10 ultrafiltration membrane.

Gel Permeation Chromatography

Ten milliliters of concentrated Concanavalin-A Sepharose eluate were dialyzed against TBS 0.1% Triton X-100, pH 7.4, 1 L volume with 4 changes buffer. After dialysis for 8 hours the material was applied to a 120 x 1.5 cm column of AcA 44 Ultrogel pre-equilibrated with TBS 0.1% Triton X-100. The column was developed isocratically at a flow rate of 6 ml/hr. One milliliter fractions were collected. Fractions were relipidated and assayed for tissue

factor activity. Peak fractions were pooled to a final volume of 20 ml. This material was stored at -20°C prior to use.

Purification of Tissue Factor Protein

Tissue factor protein was partially purified from bovine brain by a combination of acetone delipidation, Triton X-100 extraction, lectin affinity chromatography, and gel permeation chromatography. The highly purified tissue factor protein was 12,000 fold purified from brain powders (Table 1). A sensitive chromogenic assay for tissue factor protein was utilized to monitor purification steps. Following detergent extraction of acetone brain powders, the tissue factor protein activity could not be detected in the assay unless tissue factor protein was relipidated. The material which was infused into the rabbits had no cofactor activity prior to relipidation in either the one stage coagulation assay or the two stage chromogenic assay described below (Table 2). This confirmed the well known phospholipid dependence of tissue factor. See Nemerson, Y., supra. Human placental tissue factor was isolated using known methods, for example, see Guha, A. et al. supra. Human placental tissue factor protein was compared to bovine tissue factor protein. As shown in Table 5, both human placental tissue factor and bovine tissue factor have a lipid requirement for activity in an in vitro chromogenic assay. As discussed above, human placental and bovine tissue factors are similar in structure. Thus, human placental tissue factor would be expected to function similarly to bovine tissue factor if infused into rabbits.

Assay for Tissue Factor Protein

1. Chromogenic tissue factor assay.

All samples extracted from bovine brain by non-ionic detergent were relipidated prior to assay. As discussed above tissue factor has an absolute requirement for phospholipid to exhibit activity in in vitro assay systems (Pitlick and Nemeson, Supra). Lecithin granules were homogenized in Tris 0.05 M, 0.1 M NaCl pH7.4 (TBS) containing 0.25% sodium deoxycholate to a concentration of 1 mg/ml.

This solution (PC/DOC) was used to relipidate tissue factor as follows. Tissue factor protein was diluted into TBS containing 0.1% bovine serum albumin (TBSA). Fifty microliters were placed in a 12x75mm polystyrene test tube and 50 μl PC/DOC solution was added. Three hundred and fifty (350) microliters TBSA were then added along with 25 μl 100 mM $CdCl_2$. This relipidation mixture was allowed to incubate at 37°C for 30 min.

For the chromogenic assay, relipidated tissue factor protein samples were diluted in TBSA. Ten microliters were placed in a test tube with 50 μl of the factor IX_a/factor X reagent and 2 μl of a solution of purified factor VII, 30 units/ml. The tubes were warmed to 37°C and 100 μl 25mM $CaCl_2$ were added. Samples were incubated for 5 min. at 37°C prior to the addition of 50 μl chromogenic substrate S2222 containing the synthetic thrombin inhibitor I2581. The reaction was allowed to proceed for 10 min. and was stopped by the addition of 100 μl 50% glacial acetic acid solution. Absorbance was detected at 405 nM. A standard curve was constructed using rabbit brain thromboplastin (commercially available from Sigma, St. Louis, MO. catalogue #T0263) arbitrarily assigning this reagent as having 100 tissue factor units/ml. Dilutions were made from 1:10 to 1:1000. Absorbance was plotted on the abscissa on semilog graph paper with dilution of standard plotted on the ordinate.

2. One stage assay for tissue factor activity.

100 μl haemophilic plasma were added to 10 μl of relipidated or lipid free tissue factor or TBSA as control in a siliconized glass tube to prevent non-specific activation through the contact phase of coagulation. The reactants were warmed to 37°C and 100 μl 25 mM $CaCl_2$ were added and clot formation timed. Hvatum, Y. and Prydz, H., Thromb. Diath. Haemorrh. 21, 217-222 (1969).

Example 2

Efficacy and Lack of Toxicity of

Tissue Factor Protein in a Rabbit Model

Arterial and venous cannulas were inserted into the ears of two 1.8 kg New Zealand white rabbits. 0.8 ml arterial blood was withdrawn from each animal and anticoagulated with 0.2 ml 0.13 M trisodium citrate. Both animals were then infused with 600 μl protein-A purified, human, anti-human factor VIII antibody, 1700 BU/ml, through the venous cannula. Thirty minutes after the infusion, arterial cannulas were flushed with 1 ml saline and 1 ml of blood was withdrawn and discarded. 0.8 ml of blood was then anticoagulated for assay as described above. Three hundred microliters TBS/0.1% Triton X-100 was then infused into the first rabbit as a control while the second rabbit received 300 μl of tissue factor protein. On relipidation, this would represent a dose of 233 tissue factor units per kilogram (U/kg). Sixty minutes after the infusion of the antibody, blood was withdrawn from both rabbits for assay and the arterial cannulas were removed. Blood was collected and flow and duration of blood flow recorded.

Rabbit factor VIII cross-reacted with human anti-human factor VIII antibodies in in vitro assay systems. These antibodies were then used to anticoagulate rabbits thus allowing the demonstration of tissue factor protein's factor VIII by-passing activity in vivo. Thirty minutes after the infusion of anti-factor VIII antibodies, no factor VIII was detected in the plasma by chromogenic factor VIII assay (Table 3). The control rabbit received an infusion of buffer containing 0.1% Triton X-100 30 min. before the removal of the arterial vein cannula. This resulted in profuse bleeding which took eleven min. to cease (Table 3). The animal receiving tissue factor protein (test #2, at Table 3) bled only slightly after the same treatment and this flow stopped after 38 seconds demonstrating that tissue factor protein by-passes factor VIII activity in vivo. The animals receiving tissue factor protein had no observed thrombi as

had been reported in the literature and discussed above.

The toxicity of the tissue factor protein preparation was tested in six rabbits that were infused with 250 units of tissue factor protein per kilogram. After three days, no adverse effects were observed (Table 4). It should be noted that this is the dose used in Table 3 wherein the bleeding defect was corrected. Two of the rabbits were then infused with a second dose of 250 U/kg, one received twice this dose, and one rabbit received 5 times the dose. These animals, as well as two that did not receive a second injection, were monitored for an additional two days. All animals appeared normal after a total of 120 hours of observation, demonstrating that the material is well tolerated and not toxic. Similar preparations of human tissue factor protein would therefore be expected to be well tolerated when infused into patients (Table 4) and be able to correct bleeding disorders (Table 3).

Tissue factor protein is infused into hemophilic dogs using the procedure of Giles, A.R. et al., Blood 60, 727-730 (1982).

Example 3

Functional Homology Between Bovine and Human Tissue Factor Proteins

Functional homology between bovine and human tissue factor proteins was shown using the chromogenic tissue factor assay. Bovine tissue factor protein was purified as described above. Human tissue factor protein was partially purified from placentae using the method of Freyssinet et al., Thrombosis and Haemostasis 55(1):112-118 (1986) including affinity chromatography on Concanavalin-A Sepharose. The eluted material from this column was then subjected to gel filtration chromatography on an AcA 44 Ultrogel column as described earlier for the bovine protein.

Bovine and human tissue factor proteins (referred to as BTFP

and HTFP respectively in Table 5) were assayed in the standard chromogenic tissue factor assay already described. Samples that had been relipidated prior to assay exhibited potent tissue factor cofactor activity (referred to as BTFP + Pl and HTFP + Pl respectively in Table 5). Samples that had not been relipidated did not show cofactor activity in the assay (BTFP - Pl and HTFP - Pl).

Protein concentrations in these samples were bovine tissue factor protein 0.59 mg/ml and human tissue factor protein 13.55 mg/ml. The difference in protein concentration was a result of differences in the degree of purification. These results are evidence of the functional homology between the tissue factor proteins from human and bovine sources.

Table 1

Purification of Bovine Brain Tissue Factor

Sample	Vol. ml.	Protein mg/ml	Protein Total	Tissue Factor Activity U/ml	Tissue Factor Activity Total	Sp.Act. U/mg	Purification Fold
Acetone Brain Powders	3,500	7.4	25,725	1.06	3,675	0.1	-
TBS Wash Supernatant	3,000	6.0	18,120	0.16	480	-	-
0.1% Triton Supernatant	3,000	1.4	4,260	0.52	1,560	-	-
2% Triton Extract	2,750	3.0	8,250	14.82	40,761	4.9	35
Con A Sepharose Supernatant	2,750	2.4	6,600	4.2	1,133	-	-
Con A Sepharose Eluate	420	0.2	71.4	53.5	22,470	314.0	2,242
Con A Eluate Post Concentration	15	1.5	23	750.0	11,250	489.0	3,492
Ultrogel AcA 44 Pool	7.7	0.8	6.3	1,400	10,780	1,711.0	12,211

Table 2

Characterization of Partially Purified Tissue Factor Protein

Sample	Chromogenic Assay U/ml	Clotting Time Secs.
TBS/0.1% Triton buffer	0	250
Tissue Factor Protein	0	249
Relipidated TFP	1,400	66.2

Table 3

Results of *in vitro* Tissue Factor Protein Bleeding Correction

No. Rabbit	Infusion	Factor VIII U/ml Pre	30 min.	60 min.	Bleeding Time(min)	Vol.
1. Control	TBS/TX100	5.0	0	0	11.0	15.2
2. Test	TFP 233 U/kg*	4.8	0	0	0.63	0.125

* 233 U/kg of tissue factor activity after relipidation as measured in the chromogenic assay.

Table 4

Survival after infusion of Tissue Factor Protein

No.	Wt (kg)	Time 0 Infusion of TFP* Total U	Time 0 Infusion of TFP* U/kg	72 Hours Infusion of TFP* Total U	72 Hours Infusion of TFP* U/kg	120 Hours Survival (+/-)
1	1.42	350	246	350	246	+
2	1.35	350	260	350	260	+
3	1.40	350	250	700	500	+
4	1.33	350	263	1,750	1,316	+
5	1.41	350	248	0	0	+
6	1.23	350	285	0	0	+

* Units were determined by chromogenic assay after relipidation of tissue factor protein samples.

Table 5

Functional Homology between Bovine and Human Tissue Factor

Sample	Assay Dilution	Absorbance 405 nm	Tissue Factor Activity U/ml
BTFP + Pl	500	0.785	800
BTFP + Pl	1000	0.395	755
BTFP - Pl	10	0.000	0
HTFP + Pl	500	0.892	950
HTFP + Pl	1000	0.491	910
HTFP - Pl	10	0.000	0

Claims:

1. The method of treating an animal with a bleeding disorder characterized by a tendency to hemorrhage comprising administration of a therapeutically effective dose of tissue factor protein to an animal having such a bleeding disorder.

2. The method of claim 1 wherein the bleeding disorder is associated with a deficiency of a coagulation factor.

3. The method of claim 2 wherein the deficient coagulation factor is a deficiency of factor VIII.

4. The method of claim 2 wherein the deficient coagulation factor is a deficiency of factor IX.

5. The method of claim 2 wherein the deficient coagulation factor is a deficiency of factor XIII.

6. The method of claim 2 wherein the deficient coagulation factor is a deficiency of factor XI.

7. The method of claim 1 wherein the bleeding disorder is an acquired coagulation disorder.

8. The method of claim 1 wherein the tissue factor protein is administered intravenously.

9. The method of claim 1 wherein the tissue factor protein is administered orally.

10. The method of claim 1 wherein the therapeutically effective dose is in the range of about from 25 U/kg to 75 U/kg.

11. The method of claim 1 wherein the therapeutically effective dose is in the range of about from 35 U/kg to 65 U/kg.

12. The method of treating an animal with a hypercoagulative bleeding disorder comprising administration of a therapeutically effective dose of tissue factor protein antagonist to an animal having such a bleeding disorder.

13. A therapeutic dosage form for administration to an animal with a bleeding disorder characterized by a tendency to hemorrhage comprising tissue factor protein and a pharmaceutically acceptable vehicle.

14. The dosage form of claim 13 wherein the amount of tissue factor protein is from about 1750 U to 5250 U.

15. The dosage form of claim 13 which is sterile.

16. The dosage form of claim 13 which is isotonic to blood.

17. The dosage form of claim 13 wherein the vehicle is a lipophilic sustained release formulation.

18. A therapeutic dosage form for administration to an animal with a bleeding disorder characterized by a hypercoagulative state comprising a tissue factor protein antagonist and a pharmaceutically acceptable vehicle.

Abstract

A method and therapeutic composition for the treatment of bleeding disorders, for example those characterized by a tendency toward hemorrhage or a hypercoagulative state, by the administration of tissue factor protein or antagonists thereof.